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AXIAL FILAMENT OF SILICIOUS SPONGE SPICULES, ITS ORGANIC COMPONENTS AND SYNTHESIS

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The rigid skeletal structures of the marine sponges in the class Demospongiae are silicious spicules bound together by protein fibers of collagen and spongin (Hyman, 1940). The regulation of growth and form in these animals involves controlling the synthesis and placement of these spicules. There is evidence that the growth pattern, while characteristic for a taxonomic group, is yet very sensitive to environmental factors related to current directions, velocity, and turbulence (Bidder, 1923). The production of spicules *de novo* is an intracellular secretory process which has received relatively little attention (Schröder, 1936; Jørgensen, 1944, 1947). We approach the problem of sponge spicule formation as a specific cellular activity which forms the basis of multicellular morphogenetic processes. To begin the analysis of spicule growth, the paper provides a procedure for monitoring silicious deposition by means of determinations of protein metabolism. I shall present evidence supporting two conclusions. (1) The axial filament in sponge spicules has a substantial protein component. (2) Synthesis of that protein occurs during spicule growth, and may be monitored by a radioisotope label in a protein precursor which becomes incorporated into the axial filament.

The desirable isotope, ^{32}Si, is not commercially available for radio-tracer studies of silicon metabolism. If (a) spicules could be freed of external organic matter, and if (b) synthesis of any intraspicule organic matter were correlated with silicious deposition, and if (c) that organic matter were largely protein, then (a) any organic matter in the HF-residue would be intrinsic intra-spicule material, and (b) any measure of synthesis of that material would be correlated with silicious deposition, and (c) protein precursors would label it. Thus, accumulation in spicule residue of label from a protein precursor could serve as an index of silicic acid deposition during spicule growth.

In the present work harsh cleaning procedures that would remove both organic and mineral contaminants from spicules prior to HF digestion, were coupled with two kinds of experiments that are reported here; (1) determination of the organic component of axial filament, (2) demonstration of incorporation of an appropriate precursor radioisotope into the axial filament in intact and cell suspensions of sponge.

Materials and Methods

Preparation of spicules

The spicule preparation technique is based on several early observations: (1) that after digestion with HF, a microscopically visible filament remains (Bütschli, 1901); (2) that boiling concentrated nitric acid digests and dissolves protein and

other organic matter from glass; (3) that the density of spicules (1.93) is intermediate between that of organic matter (*e.g.*, DNA, 1.7) and the more common mineral contaminants (*e.g.*, quarts, 2.4) (Vosmaer and Wijsman, 1905). The procedures leading up to carbon determination were designed to have no contact with carbon containing reagents (not even acetone or alcohol) to preclude any contamination with carbon residue. (The only exception is the use of the teflon filter after carbon had been detected on gold grid preparations.) For the analysis of axial filament composition, axial filaments were prepared from chunks of sponge (*Acarnus erithacus*) from the Pacific Coast near Los Angeles (Pacific Bio-Marine, Venice, California). This species was chosen because of its relatively high content of spicules and presence of a unique "palm tree" (cladostrongyle) spicule.

Pieces of *A. erithacus* were repeatedly heated for an hour to 85–90° in changes of concentrated nitric acid until the spicules were brilliant white and the acid did not discolor (usually 4 changes). The pellet of spicules was water washed. These spicules did not darken sulfuricdichromate (standard glass cleaning solution). Density gradients were prepared in 50 ml glass centrifuge tubes using $ZnCl_2$. A linear gradient was produced by mixing two stocks, one at density 2.1, the other at density 1.8, based on standard density tables for $ZnCl_2$ (Weast, 1961). A pilot run showed that the spicules banded at the position expected for density 1.93. Preparative density gradients of 35 ml were loaded with 5 ml of spicule slurry, *i.e.*, water wet spicules taken up in density 1.7 $ZnCl_2$. The gradients were centrifuged 30 minutes at 2000 rpm in a model PR2 centrifuge. The spicules were removed with the density 1.9 to 2.0 region, and repeatedly washed by centrifugation in hot water.

Preparation of spicule residues

Cleaned spicules were etched in three different ways for micro-analysis. (1) Spicules were partially etched as previously described (Schwab and Shore, 1971) on a paladium chip. (2) Spicules were loaded on a 400 mesh gold grid 3 mm diameter and etched completely in HF. The fluid was removed by evaporation providing a "whole non-volatile residue," or by suction (supported on a membrane filter) providing an "insoluble residue." (3) Gram quantities were etched in 12 x HF in teflon weighboats, filtered with suction on weighed teflon filters, and washed with suction on weighed teflon filters, and washed with suction in 1 x HF on the same filters, providing a "washed, HF-insoluble residue." The wash water was collected for analyses which are included in a separate report.

Preparation of samples for electron microprobe

Partly etched spicules on a coded palladium chip were coated with paladium by rotary vacuum evaporation. Spicule residues on 400 mesh gold grids were similarly coated with paladium or with aluminum. The grids were held on a coded copper chip by spring clips. The chips of copper and of paladium were placed in standard specimen holders and examined in an A. R. electron microprobe. The work reported here is based on operation of the probe in 4 modes: (1) secondary electron scattering image, (2) specific wave length x-ray image, (3) specific wave length x-ray intensity scan and (4) specific wave length x-ray point-count

using a diamond standard for carbon. Qualitative inorganic analysis by means of x-ray spectral scan and specific wave length point counts are to be reported separately.

Preparation of sponge for isotope incorporation

Fingerlets of *Hymeniacidon sinapium* were aquarium-grown from sponge chunks collected in southern California (Pacific BioMarine, Venice, California) as previously described (Shore, 1971). Two modified synthetic sea-water media were used, CMF/Si and IOSW/Si. The former is based on a calcium and magnesium free balanced saline (Humphreys, 1963). The latter is based on a proprietary mixture (Instant Ocean Sea Salts made to density 1.023, Aquarium Systems, Wyckliff, Ohio). Both were made 0.5 mM with respect to silicate by the addition of $NaSiO_3$.

Cell suspensions were prepared from chunks of *H. sinapium* by mechanical disruption, and fractionated by isopycnic centrifugation in isosmotic sucrose. Sponge pieces, freed of large contaminants were sliced to pieces about 1–5 mm^3 and squeezed through nylon bolting cloth in CMF/Si, 15 g damp sponge to 30 ml medium providing about 36 ml of suspension, the "whole" cell preparation. Portions of 5 ml suspension were layered over 30 ml sucrose gradients. These were linear between CMF/Si (density 1.023) and sucrose in distilled water (density 1.092). The concentration of sucrose was calculated to be isosmotic with sea water (density 1.023) from standard tables (Wolf and Brown, 1970). After 15 minutes centrifugation at 900 rpm in PR2 three fractions were collected. These are referred to as light cells, intermediate cells, and dense cells. They are, respectively, the top 10 ml, next 10 ml, and pellet. Each of these fractions was diluted in IOSW/Si before being suspended in IOSW/Si for incubation.

Isotope incorporation

Fingerlets or cell suspension were incubated in 35 mm petri dishes (Falcon Plastics) in a water bath at 18° C. Each dish contained 3 ml IOSW/Si. Radioactivity was added as a change of medium to which had been added 3H-1-leucine, specific activity 40 Ci/mM (Schwartz Bio-research, Orange, New Jersey) at a final activity of 4 μCi/ml. All fingerlets or cell suspensions in an experiment began the incubation at 18° at the same time. The incubation in isotope occurred for periods ranging from 15 minutes to 64 hours within the incubation at 18°. The schedule routinely provided for samples which had a short isotope incubation period during the first portion and others the last portion, of the longer incubation period. For example, there was a first-hour sample and a last-hour sample during the 64-hour incubation period. Incorporation was stopped by freezing on dry ice. A first extract, containing nucleic acids and amino acids, was obtained with 15 volumes of phenol-cresol (Kirby, 1968). Cleaned, non-radioactive spicules were added to cell suspension preparations at this point in extraction. After acetone washing, the phenol-cresol residue was digested in hot nitric acid to provide a second extract. The nitric acid residue was washed in water and digested in 1 N HF providing a third residue. Portions of the medium, the first and second extracts and the third residue were then counted in dioxane-fluors (Wannemacher,

Banks and Wunner, 1965), in a liquid scintillation spectrometer (Nuclear Chicago, Mark II), to an accuracy of better than 5% at the 95% confidence level.

Amino acid analysis

A portion of a preparation of washed, HF-insoluble residue was dissolved in alkali and analyzed for protein according to Lowry, Rosebrough, Farr and Randall (1951). Portions of the same material were then taken up in water and dialyzed against water. One of these was subjected to oxidation with performic acid for 1 hour at 0° C and prior to hydrolysis. The others were directly hydrolyzed in 6 N HCl for 24 hours at 119 ± 1° C. The hydrolysates were each separated by ion exchange chromatography on a Beckman Amino Acid Analyzer. The ninhydrin-positive peaks were identified and the molar quantity of ach amino acid was calculated for the half-width by comparison with standards.

Results

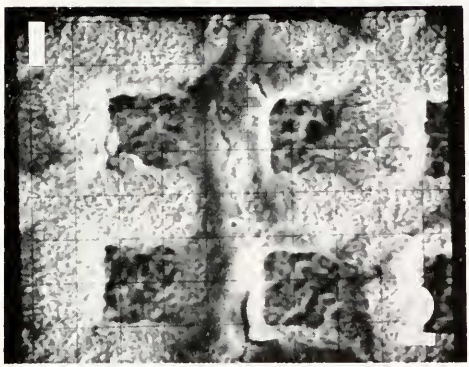
The results are presented in two sections, first the direct studies of organic composition, and second, the incorporation studies.

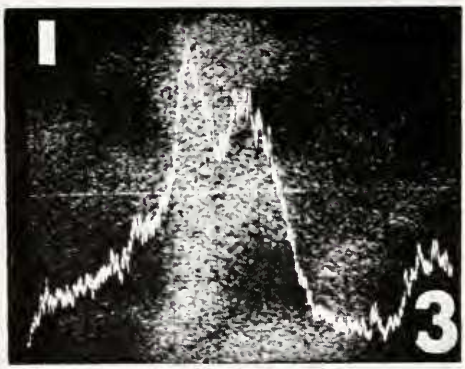
Organic composition of axial filament

Single spicules, partially etched after the fashion figured in Schwab and Shore (1971) were subjected to qualitative chemical analysis by electron microprobe. Although the axial filament could be resolved in the secondary electron scattering image, Figure 1, the current required for chemical analysis destroyed these single axial filaments before carbon analysis could be reliably completed.

Piles of spicules were then digested on gold grids and the residue examined in the electron microprobe. Figures 2 and 3 show that carbon could be detected in these aluminum coated specimens. The carbon was restricted to those portions of the grid in which residue was visible either in the light-optics image or in the secondary electron scattering image shown in Figure 2. This residue contained large quantities of other elements because the non-volatile digestion products include fluorides of Na, K, Al, and traces of several other elements previously reported (Schwab and Shore, 1971).

Encouraged by the presence of carbon, bulk preparations were made of HF resistant material. The residue that was insoluble in 1N HF was collected and washed in 1 N HF on a teflon filter. This residue was found in the electron microprobe to contain regions of high carbon content as illustrated by Figure 4 and 5. A point count of this region indicated the presence of up to 40% C. The electron scattering image confirmed that this region was an irregular pile of filamentous material with the dimensions of axial filaments or clusters of these. Individual threads about the diameter of axial filaments may be seen in Figure 4 in the region giving this high carbon content. A second bulk preparation was made to provide material for quantitative organic analysis. From 6.89 g of purified spicules, the total non-volatile residue after digestion in 12 N HF, collected on teflon, was 596 mg. Of this, the fraction insoluble in 1N HF, collected on a teflon filter, was 12.4 mg. Determination of protein by the method of Lowry *et al.*





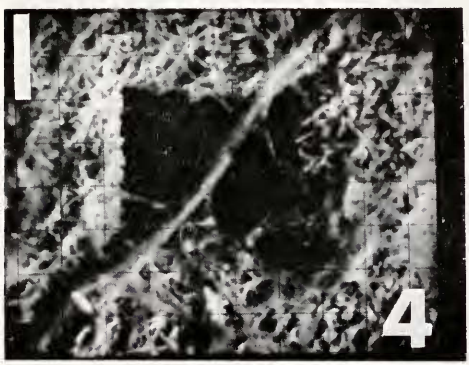


FIGURE 1. Secondary electron image of a single, partly etched style with HF resistant axial filament, scale line = 20 μ.

FIGURE 2. Secondary electron image of spicule residue on gold 400 mesh grid.

FIGURE 3. Carbon x-ray image of the field shown in Figure 2, with superposed plot of intensity of these x-rays (ordinate *vs.* distance).

FIGURE 4. Secondary electron image of washed spicule residue on gold 400 mesh grid.

FIGURE 5. Carbon x-ray image of the field shown in Figure 4.

TABLE I

Mole per cent composition of axial filament protein

Residue	Mole%	Residue	Mole%	Residue	Mole%	Residue	Mole%
Asp/Asn	14	Ala	6.8	Pro	5.0	His	2.7
Gly	11	Ser	6.3	Phe	5.0	Arg	2.8
Val	10	Leu	6.3	Lys	3.7	Tyr	1.4
Glu/Gln	9	Ile	5.9	Cys	2.9		
		Thr	5.5	Met	2.9		

(1951) on $\frac{1}{4}$ of this filter gave a color equivalent to 900 μg protein. That indicates the presence of about 3.6 mg protein in the 12.4 mg residue for about 29% protein. This 12.4 mg residue had the characteristic lavender color we have noted before in preparations of spicule residue. In HF or upon drying from HF, the residue is lavender. Water washing changes the color reversibly to a straw tan.

The results of the determinations of amino acid composition are summarized in Table I. When the initial separations were made using the Lowry value for protein content, the quantity of amino acids recovered was so far below that estimated that the chromatograph could not be used for a quantitative indication of the amino acid composition. A pair of determinations was then made using larger quantities of material. The most reliable estimate of the values for methionine and cysteine are believed to be those based on the pre-oxidized sample from which methionine is recovered as the sulfon and cysteine as cysteic acid. The paired values to other amino acids are within one mole per cent of each other, except as expected for methionine and cysteine. The major components of axial filament protein are aspartic (including asparagine), glycine, valine, and glutamic (including glutamine). There is no detectible hydroxyproline or hydroxylysine. Tryptophane would have been destroyed by the hydrolysis so it remains totally unestimated.

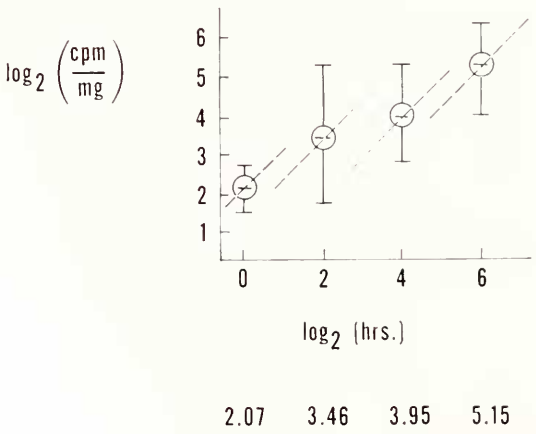


FIGURE 6. Time course of 3H-leucine incorporation into bulk protein for fingerlets of *Hymeniacidon sinapium*.

Incorporation studies

The incorporation of tritium from l-leucine into protein in sponge tissue occurs at 18° C. A determination of that incorporation as a function of duration of incubation is presented in Figure 6. The accumulation of incorporated radioactivity increases with time at least up to 64 hours under the conditions of incubation. The rate of accumulation decreases with increasing time in the presence of radioactive precursor. This may be seen from the slope in the figure. The dotted lines indicate the incorporation required for constant incorporation rate. However, each of the time points (except the 64 hour point) was based on both early and late replicate fingerlets. Furthermore, the rate of incorporation for *both* early and late 1-hour incubations is greater than the mean incorporation rate for 4-hour incubations. Therefore, this falling incorporation rate is not the result of a falling

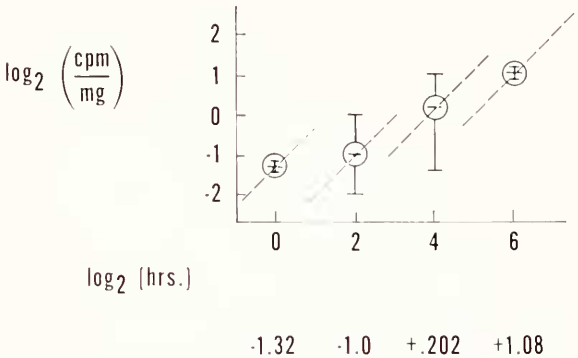


FIGURE 7. Time course of 3H-leucine incorporation into axial filament for fingerlets of *H. sinapium*.

biological synthesis rate but rather is more likely the result of a falling specific activity of precursor pools. Thus the conditions of incorporation underestimate but do not inhibit protein synthesis during prolonged incorporation into intact sponge fingerlets.

Some incorporation has been detected into what is believed to be axial filaments. Tritium from leucine, equal to a small fraction of that incorporated into bulk protein, is incorporated into the HF non-volatile residue of spicules. The procedure of HF digestion leaves the axial filament morphologically without change, but completely removes organic matter from the spicule surface prior to HF digestion. The time course of incorporation of tritium into this non-volatile spicule residue in intact sponge fingerlets is shown in Figure 7. The incorporation increases with time. The rate of incorporation appears to be gradually decreasing with duration but there is no significant correlation between the rate of incorporation and the duration of the pre-incorporation incubation.

Finally the incorporation of tritium into the HF-insoluble residue was measured for cell suspensions. The data in Table II show the radio-activity that is not removed from spicules by repeated nitric acid digestion in the presence of added "carrier" spicules, nor subsequently made soluble by digestion and washing on a

filter with 1 N HF. Statistically significant incorporation occurred in both "intermediate" and "dense" cell preparations. No incorporation was detected in either the "whole" or the "light" cell preparation.

Discussion

These data on the composition of axial filament are direct evidence that protein is a constitutent of the axial filament of silicious sponge spicules. This confirms the conclusion of many workers based on a large body of indirect evidence. The most important early work was Bütschli's exhaustive testing of spicule residues and microscopic observation of axial filament, in various reagents (Bütschli, 1901). Others have noted the presence of an axial filament resistant to digestion in HF (deLaubenfels, 1932; Lévi, 1963; Minchin, 1909; and Travis, Camille, Bonar and Glimcher, 1967). The most convincing evidence of a substantial protein com-

Table II

Radioactivity of axial filament fraction after 3-H-leucine incorporation by cell-suspension preparations of Hymeniacidon sinapium. *Axial filament fraction was the residue after (a) digestion in 3 changes of hot conc. HNO_3 in the presence of "carrier" spicules, (b) digestion in 1 N HF, (c) membrane filter washing in 1 N HF. Radio-activity determined on filters in toluene-flours by liquid scintillation.*

Cell preparation	Incubation condition		
	With 3-H-Leucine	IOSW alone	Difference
Light	17.7 ± 0.72	17.6 ± 0.72	0.1 ± 1.4
Intermediate	23.8 ± 0.96	15.1 ± 0.60	8.7 ± 1.4
Dense	20.4 ± 0.80	13.4 ± 0.56	6.8 ± 1.3

ponent in axial filament comes from Garrone (1969). By digestion in selected enzymes he showed that a protease-sensitive material is required for the structural integrity of the axial filament. This enzymatic evidence that protein was an important component of the axial filament of sponge macroscleres provided no estimate of the fraction of axial filament that was protein. We had direct evidence from combustion and gas chromatography that there was sufficient carbon in the residue from HF digestion of spicules to provide a structure of the size of the axial filament and a composition of 40% C as it would be if solely protein or carbohydrate (Schwab and Shore, 1971). I, therefore, wished to determine the position in the spicule and the chemical form of that carbon.

From earlier analyses (Schwab and Shore, 1971) we inferred that carbon was present in the bulk axial filament residue, enough to provide up to 40% of the axial filament as protein or carbohydrate. From the microprobe data presented here, we may infer that the only detectable concentration of carbon occurs in the spicule residue in the position of individual axial filaments. From the Lowry assay datum we may infer that some protein is present in that residue, perhaps as much as 30% of the residue. From the amino acid analysis data we may infer that a

material with the composition of protein is present in the residue and represents upwards of 8% of the Lowry-positive material, or upwards of 2% of the HF-insoluble residues. We have thus set a lower limit on the proportion of protein in the axial filament. The conclusion, that upwards of 2% of the axial filament is protein, is consistent with Garrone (1969). There is an apparent discrepancy between this conclusion and that of Drum (1968) and of Travis *et al.* (1967), namely, they conclude that the axial filament is carbohydrate. The relative proportions of protein and carbohydrate in axial filament and their manner of combination remain as yet unknown.

A large body of theory exists dealing with the manner in which specific protein synthesis is regulated (see for example the volume summarized by Lengyel (1969). In order to bring this theory to bear on the control of spicule growth it would be sufficient to show that specific protein synthesis is associated, in a non-trivial way, with spicule growth or more strongly, with silicious deposition in spicules. The data presented above support the conclusion that there is an association in both time and place between on the one hand, the incorporation of a protein precursor into some material associated with protein and, on the other, the deposition of apparently silicious material. The deposition is at least sufficient to protect the protein and the incorporated label from digestion by boiling nitric acid.

There remain yet to be determined the degree to which the label in the axial filament is actually in leucine, in protein, in a specific protein, or in one required for silicious deposition. This last point is the thrust of experiments to be undertaken next.

We have thus provided a procedure by means of which we may now ask about the relationship between protein synthesis (and incorporation into axial filament) and silicious deposition (in spicules) in the analysis of sponge growth and development. The present experiments thus make possible the experimental posing of questions which are formulated in terms of mechanisms regulating protein synthesis and which bear directly on the mechanism of regulation of silicious deposition.

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Summary

1. A substantial portion of the axial filament of the spicules of silicious sponges is protein.

2. The axial filament protein lacks the hydroxylated amino acids that are characteristically abundant in collagen.

3. It is feasible to follow the course of silicious deposition in spicules using C^{14} or H^3 in protein precursors.

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